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Reduction of Water Repellence of Hydrophobic Plant Substrates Using Biosurfactant Produced from Hydrolyzed Grape Marc

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This work demonstrates that the biosurfactant produced by *Lactobacillus pentosus* from grape marc hydrolysates can be successfully employed in reducing the water repellence of hydrophobic substrates, rather than chemical surfactants, as it can be produced from low-cost residual materials and it is less toxic than chemical surfactants. The method employed to measure the water repellence of the 11 plant substrates, consisting of pine bark, peat, and composts from various origins (biodegradable fraction of municipal solid waste, green waste, sewage sludge, manure, pine bark, and grape marc), was the molarity of ethanol droplet method (MED). Peat, pine bark, and the composts obtained from grape marc and pine bark were severely hydrophobic, having contact angles over 104°, whereas the composts from municipal solid waste were less hydrophobic, with contact angles under 101°. When hydrophobic substrates were treated with the biosurfactant from *L. pentosus*, the water repellence of the plant substrates was reduced in all but two cases (the least hydrophobic composts), achieving in most of the cases results better than those obtained using chemical surfactants.

KEYWORDS: Water repellence; grape marc; biosurfactant; plant substrates; compost

INTRODUCTION

Many organic materials used as plant substrates, such as peat or pine bark, develop hydrophobic properties after drying, with effects on their water-holding capacity and rewetting time and the patterns of water supply to plants (*1*, *2*). The application of surfactants has been proved suitable for reducing water repellency in soils (*3*), so the same strategy could be used for organic substrates. When a surfactant is added to air/water systems at increasing concentrations, a progressive reduction of the surface tension is observed until it reaches a concentration that renders the minimum surface tension value. Above this concentration, known as the "critical micellar concentration" (CMC), it is not possible to continue lowering the surface tension due to the fact that surfactant molecules readily associate to form supramolecular structures such as micelles and vesicles. Many processes rely on conventional surfactants to increase the wettability of hydrophobic substances. For instance, organic surfactants are added to herbicide solutions to achieve a larger wetted area when the solution is applied to the leaf of a plant (*4*). Microbial surfactants, named biosurfactants, have several advantages over chemical surfactants, including lower toxicity and higher biodegradability and effectiveness at extreme temperatures or pH values (*5*, *6*). These biosurfactants/bioemulsifiers can be produced in the cell-free extract or linked to their plasmatic membrane. Portilla et al. (*7*) reported that *Lactobacillus pentosus* produces cell-bound biosurfactants when grown in grape marc hydrolysates, which could be cost competitive with chemical surfactants. Moreover, biosurfactants from *L. pentosus* not only reduce the surface tension of the media but also have emulsifying properties that could be useful to reduce the water repellence of hydrophobic substances (*8*).

Despite their relevance for water management in growing media, the hydrophobic properties of plant substrates are not usually analyzed, although they are made up of organic substrates that may present an important degree of water repellence (*1*, *2*, *9*), and specific methods for its determination are not established in the European Standards. Leelamanie et al. (*10*) compared the performance of various methods to measure the soil−water repellence, the water drop penetration time (WDPT) method, the molarity of ethanol droplet (MED) method, the capillary rise method (CRM), and the sessile drop method (SDM), concluding that the MED test was the most suitable for materials with contact angles ≥90°. Plant substrates have been reported to present contact angles >90° by other workers (*1*), so the MED test would be the most accurate to determine the contact angle in these substrates. Moreover, the MED test is a simple and rapid method for assessing soil−water repellency under field and laboratory conditions (*11*), and it does not require complex equipment for its measurement.

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Consequently, the objectives of this work were (i) to evaluate the hydrophobic properties of several organic substrates used for the elaboration of plant growth media and the suitability of the MED test for this task; and (ii) to determine if biosurfactants, obtained from *L. pentosus* by fermentation of hemicellulosic sugars from grape marc, could be employed to reduce the water repellency of the plant substrates in comparison with chemical surfactants.

MATERIALS AND METHODS

Plant Substrates. Table 1 shows the nomenclature and origin of the substrates evaluated in this work. MSW1 is a compost obtained by anaerobic fermentation of the biodegradable fraction of municipal solid waste (MSW), separated before collection, followed by an aerobic composting step, to stabilize the incompletely digested residue, whereas MSW2 is an aerobic compost obtained from the source-separated biodegradable fraction of MSW. Both MSW composts were provided by industrial composting facilities in A Coruña (Spain). MSGW is a compost obtained from the source-separated biodegradable fraction of MSW mixed with green waste, and MGSS is compost from municipal garden trimmings mixed with sewage sludge, whereas MV is a mixed manure vermicompost. CPB is composted pine bark produced by aerobic composting in windrows. GMC and GMV1 are a grape marc compost and a grape marc vermicompost, respectively, and they were prepared simultaneously on a low-scale windrow in a 6 month process (*12*). Finally, GMV2 is a grape marc vermicompost prepared on an industrial scale. On the other hand, two other common constituents of plant substrates were used: noncomposted pine bark (PB) and sphagnum peat (P).

For the analysis of the general properties of substrates, the Spanish version of the European CEN methods for the characterization of soil amendments and substrates (*13*−*16*) was followed. Briefly, pH and electrical conductivity (EC) were determined in aqueous extracts (substrate/extractant ratio of 1:5 v/v) of fresh samples. Total organic matter (OM) was determined by weight loss on ignition of dried ground samples at 450 °C, and total organic C (TOC) was calculated by multiplying the OM concentration by a factor 0.58. Total N was measured by Kjeldahl digestion and steam distillation (*16*). The humification index (HI) of the substrates was determined following the Spanish official method for the extraction of humic acids in organic amendments (*17*). Half a gram of dry sample was extracted at room temperature during 1 h with 100 mL of 0.1 M NaOH + 0.1 M $Na_4P_2O_7$ solution. The extract was then centrifuged at 4500 rpm during 25 min, and the procedure was repeated twice, mixing all of the extracts and adjusting them to 1 L. The humic acid fraction was separated from the fulvic acid fraction by precipitation after acidification of the solution to pH 1, redissolved with 0.5 M NaOH, and adjusted to 50 mL with distilled water. The organic carbon content of the fractions was determined by the wet dichromate oxidation procedure, and the fulvic carbon was determined as the difference between the total alkali-extractable C and the humic acid C. The HI was calculated as the percentage of humic acid C to TOC (total organic carbon).

Biosurfactant. *Preparation of Hemicellulosic Hydrolysates from Grape Marc.* Prehydrolysis (partial acid hydrolysis of the hemicellulosic fraction) of distilled grape marc was carried out in an autoclave at 130 °C with 3% H_2SO_4 for 30 min using a liquid/solid ratio of 8 g/g (*8*).

Microorganism. *L. pentosus* CECT-4023T (ATCC-8041) was obtained from the Spanish Collection of Type Cultures (Valencia, Spain). The strain was grown on MRS broth at 31 °C for 15 h and 150 rpm.

Biosurfactant Production. Hemicellulosic hydrolysates from grape marc were neutralized with powdered $CaCO_3$ to a final pH of 6.0, and the $CaSO_4$ precipitated was separated from the supernatant by filtration. The clarified liquors were supplemented with 10 g of yeast extract L^{-1} and 10 g of corn steep liquor L^{-1} (*18*), sterilized at 100 °C for 1.25 h, and used directly as fermentation media. Next, the inoculum was centrifuged, and *L. pentosus* cells were resuspended in the same volume of neutralized hydrolysate, following the methodology proposed by Portilla et al. (*8*). Once the fermentation was finished *L. pentosus* cells were recovered by centrifugation (4500*g*, 30 min, 20 °C) from the fermentation media, washed twice in demineralized water, and resuspended in phosphate-buffered saline (PBS; 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to pH 7.4) to extract the biosurfactant bound to *L. pentosus* cells.

Commercial Surfactants. The commercial surfactants employed in this work consisted of Tween 20 (Panreac S.A., Barcelona, Spain) and ISOOP (Burés Professional S.A., Girona, Spain). Tween 20 is a chemical surfactant that is commonly used in the literature for the bioremediation of contaminated soils, due to its surfactant properties. However, to our knowledge, Tween 20 has not been tested previously for reducing the water repellence of plant substrates. On the other hand, ISOOP is one of the few products commercially available in Spain to reduce the water repellence of soils or plant substrates. Tween 20 is a polysorbate surfactant having stability and relative nontoxicity that allow it to be used as a detergent and an emulsifier in a number of domestic, scientific, and pharmacological applications. It is a polyoxyethylene derivative of sorbitan monolaurate and is distinguished from the other members in the Tween 20 range by the length of the polyoxyethylene chain and the fatty acid ester moiety. On the other hand, ISOOP is a liquid commercial humectant consisting of C8-alkyl polyglucoside, used to facilitate the penetration and distribution of irrigation water in soil and substrates. Tween 20 and ISOOP reduce the surface tension of water from 72 to 34 and 54 mN m^{-1}, respectively.

Evaluation of Surfactants' Phytotoxicity. The phytotoxicity of the surfactants (biosurfactant from *L. pentosus*, Tween 20, and ISOOP) was determined using a germination−elongation test with cress (*Lepidium sativum* L.), following the protocol used by Moldes et al. (*19*) based on the Zucconi method (*20*). Prior to the test, the surfactants were diluted to the same concentration used for the treatment of the substrates.

Evaluation of Surfactants Toxicity to Microorganisms. The toxicity of the surfactants (biosurfactant from *L. pentosus*, Tween 20, and ISOOP) to microorganisms was determined using the Microtox luminescence test. Prior to the test, the surfactants were diluted to the same concentration employed for the treatment of the substrates. The toxicity test for each solution was carried out with the bacteria *Vibrio fischeri* using a model Microtox 500 analyzer (Azur Environmental Ltd.). Each solution was serially diluted (1:2), and the luminescent bacteria *V. fischeri* were added at each dilution. The bacteria were exposed to concentrations of 45, 22.50, 11.25, and 5.62% (v/v) solution diluted with Microtox test medium (Azur Environmental Ltd.). The inhibition of luminescence was measured after 15 min, and the EC_{50-15} (concentration of the solution that produces a 50% inhibition of the luminescence after 15 min) was computed using the Microtox software.

Table 1. Properties, Nomenclature, and Origin of the Substrates Evaluated in This Work.

substrate	nomenclature	origin
municipal solid waste compost	MSW1	Albada (A Coruña, Spain)
municipal solid waste compost	MSW2	FCC (A Coruña, Spain)
compost from municipal solid waste + green waste	MSGW	Metrocompost (Barcelona, Spain)
compost from municipal garden trimmings + sewage sludge	MGSS	Metrocompost (Barcelona, Spain)
mixed manure vermicompost	MV	Comporense (Ourense, Spain)
composted pine bark	CPB	Costiña (Lugo, Spain)
grape marc compost	GMC	University of Santiago de Compostela, Spain
grape marc vermicompost	GMV1	University of Santiago de Compostela, Spain
grape marc vermicompost	GMV2	Ecocelta (Pontevedra, Spain)
noncomposted pine bark	PB	Dermont (A Coruña, Spain)
peat	P	Miksskaar AS (Estonia)

Treatment of Plant Substrates with Surfactants. The surfactants were diluted with distilled water before treatment. The biosurfactant from *L. pentosus* was diluted 1:1; Tween 20 was used at a concentration of 1 g L^{-1} (which is above its CMC), and ISOOP was used at a concentration of 0.1% v/v, 2-fold the recommended concentration for its use. Subsequently, plant substrates were treated with the biosurfactant from *L. pentosus* or with the commercial surfactant solution, using a 2:1 substrate/surfactant ratio (v/v), sufficient to achieve total soaking of the substrate with the solution, and left in contact for 48 h. After that time, the materials were dried at 60 °C and the water repellence was measured following the protocol described below.

Water Repellence Determination. The water repellence of plant substrates was evaluated by using the molarity of ethanol droplet (MED) test as described by Roy and McGill (*11*) for soils, with some modifications for the analysis of organic materials. First, the materials were dried at 60 °C to level their moisture content and ground to obtain homogeneous samples. Although the original MED test uses soil dried at 105 °C, this step was modified because vegetal materials are usually dried at 60−70 °C to preserve their structure. Aqueous solutions of ethanol in increments from 0.2 to 6 M were prepared. Plates, 65 mm in diameter, were filled with samples of each substrate and the surface leveled by shaking and tapping the dish on the benchtop. One hundred microliter droplets of each ethanol solution were poured on the surface of the compost, and the time of initial droplet entry was annotated. This procedure was repeated with solutions of increasing concentrations until the time of initial droplet entry was under 10 s. Three replicates of each sample were tested. Results were reported as molarity of ethanol and contact angle (θ) of the solution. The contact angle (θ) is an indicator of the free energy of the solid/gas interface. When θ is $<90°$, water displaces air and wets soil spontaneously, whereas if θ is $>90°$, an external force is required to force the displacement of air to wet the soil. To calculate θ, the molarity of ethanol was first converted to surface tension (γ_c) using the equation (*21*)

$$\gamma_c = 61.05 - 14.75 \times \ln(\mathrm{MED} + 0.5)$$

where MED is the lowest molarity of ethanol solution that is absorbed by the compost within the first 10 s. The surface tension of the ethanol solution was then used to calculate θ by means of the equation (*21*)

$$\cos\theta = \left(\frac{\gamma_c}{\gamma_w}\right)^{1/2} - 1$$

where γ_w is the surface tension of water (72 mN m^{-1}).

RESULTS AND DISCUSSION

General Properties of the Materials. Table 2 shows the general properties of the materials evaluated in this study. The MSW composts presented the highest pH values, and all of the other composts were near neutrality, whereas peat and pine bark were acidic. The values for OM, TOC, and total N were lower for MSW composts than for the composts from other origins and P and PB. The C/N ratio was in the same range between 14 and 22 for all composts except CPB, which was much higher at around 211. Traditionally, the limit for mature compost has been fixed at about 20 (*22*, *23*), and only three composts were above that value (MV, GMC1, and GMC2). Taking into account the HI, it follows that MSGW and MGSS are the most mature composts and the grape marc composts the least.

Water Repellence of the Plant Substrates. Table 3 shows the results of the MED test for the evaluated substrates. According to King (*24*), the water repellence of the materials can be classified according to the molarity of ethanol: materials with MED values up to 1 M have low hydrophobicity ($\theta \leq 97.5°$), materials with values between 1.2 and 2.2 M have moderate hydrophobicity ($98.3° \leq \theta \leq 101.6°$), materials with values between 2.4 and 3 M are severely hydrophobic ($102.2° \leq \theta \leq 103.6°$), and materials with MED values of 3.2 M or higher are extremely hydrophobic ($\theta \geq 104°$). The hydrophobicity of 9 of the 11 substrates could be exactly determined using this method. With regard to the other 2, MV was too hydrophilic ($\theta < 90°$) and GMC was too hydrophobic ($\theta > 109°$). Among the tested substrates, peat, pine bark, and the composts made from grape marc and pine bark gave the highest water repellence values ($\theta > 104°$), whereas the MSW composts were less hydrophobic ($96.5° < \theta < 101°$), which could be an advantage for their use in the elaboration of plant growth media. These data are in accordance with those reported by Valat et al. (*1*), who found that peat has contact angles higher than substrates consisting of composted materials.

Figure 1 shows the relationship between the organic matter concentration of the substrates and their contact angles. It can be observed that the organic matter concentration is clearly related to the water repellence. The materials with OM values of >90% (CPB, GMV, P, and PB) gave the highest contact angles. Some authors also suggested that the hydrophobic properties of substrates can be related to their organic matter content (*2*, *9*), which agrees with the observations of other authors that fresh organic wastes or low humification compounds are the

Table 2. General Properties of the Plant Substrates

	pH	EC[a] (dS m^{-1})	OM[b] (% w/w)	C (% w/w)	N (% w/w)	C/N	HI[c]
MSW1	8.4	2.3	49.0	28.0	1.7	17	15.0
MSW2	8.2	2.4	39.7	23.0	1.5	15	21.4
MSGW	9.2	1.2	42.9	24.8	1.7	14	25.7
MGSS	7.3	1.4	51.5	29.8	1.8	15	31.5
MV	7.9	0.7	37.6	21.7	1.0	21	19.6
CPB	6.2	0.4	98.1	57.0	0.3	211	5.2
GMC	7.2	0.5	95.0	55.1	2.5	22	14.3
GMV1	7.0	0.7	94.8	55.1	3.5	16	9.8
GMV2	7.8	0.4	90.9	52.7	3.0	18	8.5
P	3.9	0.02	98.8	57.3	0.7	84	
PB	5.5	0.04	97.0	56.2	0.2	230	

[a] EC, electrical conductivity. [b] OM, organic matter. [c] HI, humification index.

Table 3. Water Repellence of the Plant Substrates before and after the Addition of Surfactants

	no additive		biosurfactant		Tween 20		ISOOP	
	MED (mol L^{-1})	θ (deg)	MED (mol L^{-1})	θ (deg)	MED (mol L^{-1})	θ (deg)	MED (mol L^{-1})	θ (deg)
MSW1	2.0	101.0	0.6	95.4	<0	<90	0.2	92.6
MSW2	1.2	98.3	0.4	94.1	0.4	94.1	1.2	98.3
MSGW	0.8	96.5	1.2	98.3	1.0	97.5	0.4	94.1
MGSS	1.2	98.3	1.4	99.1	1.4	99.1	1.4	99.1
MV	<0	<90	<0	<90	<0	<90	<0	<90
CPB	4.2	106.0	3.0	103.6	3.8	105.2	4.0	105.6
GMC	>6	>109	<0	<90	<0	<90	<0	<90
GMV1	3.8	105.2	1.4	99.1	3.0	103.6	0.2	92.6
GMV2	3.4	104.4	1.8	100.4	1.6	99.8	0.8	96.5
P	5.8	108.5	4.2	106.0	5.8	108.5	5.4	107.9
PB	4.0	105.6	2.2	101.6	4.0	105.6	3.8	105.2

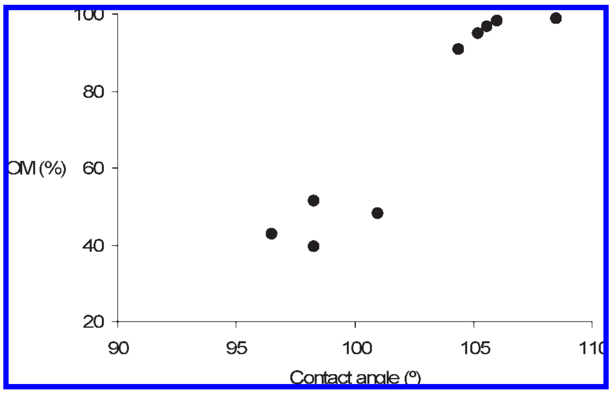

Figure 1. Relationship of water repellence and organic matter (OM) in plant substrates.

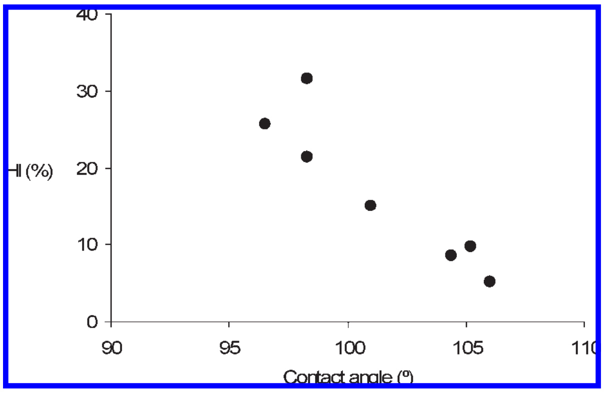

Figure 2. Relationship of water repellence to humification index (HI) in composted plant substrates.

cause of hydrophobicity in soils (*25*, *26*). Moreover, it was observed that the water repellence of the composted substrates decreased inversely to the degree of humification (**Figure 2**). Although an inverse behavior was observed for peats with different degrees of humification, Valat et al. (*1*) found that the contact angle of composts decreased during composting, in parallel with an expected increase of humification. This suggests that the utilization of noncomposted green waste or residues with low humification index as soil amendments could increase the water repellence of soils.

Evaluation of the Phytotoxicity and Ecotoxicity of Biosurfactants from *L. pentosus* Compared with Chemical Surfactants. An interesting and novel alternative to reduce the hydrophobic properties of plant substrates could consist in the utilization of biosurfactants obtained through fermentation of low-cost carbon sources such as grape marc. Some authors proposed the utilization of chemical surfactants to reduce the hydrophobic properties of soils or other substrates (*3*), but chemical surfactants are more toxic and less stable at extreme temperatures or pH values than microbial surfactants (*5*, *6*). Despite the potential benefits of biosurfactants from *L. pentosus* compared with chemical surfactants, before it is employed to reduce the hydrophobicity of plant substrates, it is important to test if it can be toxic to plants or animals. For this reason the surfactants employed in this work were submitted to a phytotoxicity test (*20*), and it was found that the biosurfactant from *L. pentosus* was not phytotoxic, as shown by the germination index, which had a value of 123%. According to Zucconi et al. (*20*), values for the germination index of < 50% indicate high phytotoxicity, values between 50 and 80% indicate moderate phytotoxicity, and values of > 80% indicate that the material presents no phytotoxicity, whereas values of > 100% are indicative of a phytostimulating effect. On the contrary, the solutions containing chemical surfactants produced reductions of the germination indices, which were 80% for the solution of Tween 20 and 69% for the solution of ISOOP, so both substances have to be regarded as moderately phytotoxic. Moreover, the results of the ecotoxicity test gave the following results for the EC_{50-15}: 47.2% for the biosurfactant from *L. pentosus*, 34.4% for the ISOOP solution, and 3.1% for the Tween 20 solution. Ecotoxicity is inversely related to the value of the parameter EC_{50}. Thus, the biosurfactant was less toxic than the chemical surfactants, even when chemical surfactants were used at high levels of dilution.

Table 4. Properties of the Biosurfactant Employed for Reducing the Water Repellence of Plant Substrates

	biosurfactant from *L. pentosus*
units of surface tension reduction (mN m^{-1})	16.2
F_{CMC}[a]	3.0
rmulsion capacity[b] (%)	83.0
dtability of emulsion after 72 h[c] (%)	99

[a] F_{CMC} consists of the dilution ratio needed to reach the critical micellar concentration of biosurfactant. [b] Emulsion capacity of biosurfactants from *L. pentosus* cells is given as the percentage of emulsified kerosene. [c] Emulsion consisted of a kerosene/water emulsion stabilized by biosurfactants from *L. pentosus.*

Effect of the Surfactants on the Water Repellence of the Plant Substrates. To our knowledge, the use of microbial surfactants to decrease the water repellence of plant substrates has not been proposed to date. The major obstacle for the wide-scale industrial application of biosurfactants is the high production cost coupled with a low production rate, as compared to commercially available synthetic surfactants (*27*). From this point of view, the utilization of grape marc hydrolysates as carbon source for producing biosurfactants can make the production cost of microbial biosurfactants competitive with the production of synthetic surfactants and, consequently, they can be employed in the water repellence correction of hydrophobic plant substrates.

Table 4 shows the surface active properties of the biosurfactant employed in this work that have already been evaluated in a previous work by Portilla et al. (*8*). Biosurfactants from *L. pentosus* can reduce the surface tension of aqueous solution from 72 to 55.8 mN m^{-1} and exhibit an additional emulsifying capacity.

The hydrophobicity of the plant substrates after the addition of the biosurfactant (**Table 3**) was reduced in all cases except for the substrates with the highest HI (MGSS and MSGW), which showed the lowest initial measurable hydrophobicity. The highest reduction took place for GMC, for which the contact angle decreased from > 109° to < 90°, whereas the contact angle of peat and pine bark decreased from 108.5° and 105.0° to 106.0° and 101.6°, respectively. When the results obtained with biosurfactant from *L. pentosus* were compared to those of the chemical surfactants (**Table 3**), it was observed that the performance of Tween 20 was superior for the composts MSW1, MSGW, and GMV2 and equal for MSW2, MGSS, and GMC, but inferior for P and PB, for which it exerted no effect. A similar behavior was observed when the substrates were treated with the commercial surfactant ISOOP. This achieved higher reductions than the biosurfactant for the composts MSW1, MSGW, GMV1, and GMV2, but it was not able to reduce the hydrophobicity of P, PB, or CPB as the biosurfactant from *L. pentosus* did.

The precise causes of the differences of performance observed between the biosurfactant and the chemical surfactants will be more deeply investigated in the future. A stimulating effect of the microbial activity induced by substances of microbial origin present in the biosurfactant could have taken place, leading to the partial degradation of the molecules being the cause of hydrophobicity in peat and pine bark, such as lipids, waxes, and resins (*28*); also, other classes of interaction could occur between the complex biosurfactant solution and the hydrophobic molecules in peat and pine bark, which could have led to the same result. In any case, these hypotheses must be confirmed or rejected by further research.

Nevertheless, this work showed that the hydrophobicity of 9 of the 11 plant substrates evaluated was accurately determined using the MED test, so it can be concluded that this method is suitable for assessing hydrophobicity in most substrates. The fact that almost all of the composted materials, as well as peat and pine bark, presented water repellence when dried at 60 °C (contact angle > 90°) shows the importance of moisture control when organic plant substrates are marketed and employed. The use of surfactants, of either chemical or microbial origin, for the reduction of hydrophobicity of plant substrates was demonstrated to be a useful strategy. The biosurfactant produced by *L. pentosus* from grape marc hydrolysates could be successfully employed in this task, rather than chemical surfactants, as it can be processed from low-cost residual materials and it is less toxic than chemical surfactants.

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